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Article Addendum

# The *Bacillus subtilis* stressosome

## A signal integration and transduction hub

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**The stressosome is a unique mediator of inducible gene expression in a wide variety of bacterial species. The 1.8 MDa stressosome complex in *Bacillus subtilis* is a key signal transducer in the environmental stress response of the bacterium, its activation leading ultimately to the upregulation of over 150 genes. The single particle cryo-EM derived molecular envelope of the stressosome was used to generate a pseudo-atomic model by fitting the crystal structures of known components of the complex. The final structure comprises three separate proteins, RsbR, RsbS and RsbT in an unusual arrangement with a pseudo-icosahedral core with sensory extensions provided by the N-terminal domain of RsbR. Immuno-localization studies of the stressosome in fixed *B. subtilis* cells showed that the complexes are located as punctate foci in the cytoplasm and are stable throughout the imposition of stress. Furthermore, we investigated the response to a number of environmental stressors and found that the response elicited by the stressosome showed a cooperative effect. Taken together, these results imply that the stressosome acts to integrate stress signals from multiple sources, and offers a tunable and co-operative response to activating signals. Our findings, as well as their implications for bacterial signaling, are further discussed in this addendum.**

Microorganisms respond to fluctuating environmental conditions by the upregulation or differential expression of genes that confer resistance to the particular stress, or enter a more general short-lived "stress-resistant" state.[1] In Gram positive bacteria such as *Bacillus subtilis* the alternative sigma factor $\sigma^B$ activates the expression of over 150 genes in response to both environmental and energy stress.[2] The stressosome acts as a signaling hub to integrate a diverse array of signals that activate the $\sigma^B$ cascade and thus the stress response.[3,4] Previous work has shown that the *B. subtilis* stressosome consists minimally of two proteins, RsbR and RsbS, which sequester a kinase, RsbT, responsible for the activation of the $\sigma^B$ cascade.[4-6] A number of paralogues of the RsbR sensor protein exist in *B. subtilis*, and these have been suggested to respond to discrete signals such as blue light, in the case of YtvA.[7-9] The RsbR-S-T component of the so-called Regulator of sigma B (Rsb) operon is found in many microbial phyla, including, but not limited to, the Methanomicrobiales branch of the Euryarchaea, Proteobacteria, Fermicutes, Cyanobacteria, Actinobacteria and members of the *Bacteroidetes* and *Deinococcus* groups.[3] The downstream chromosomal organization in these organisms points to the recruitment of stressosome orthologs to the regulation of aerotaxis, biofilm formation and secondary messenger biosynthesis. It would thus appear that the stressosome has been widely adopted to solve the problem of signal integration.

The *B. subtilis* stressosome was originally identified as a 1.5 MDa complex of approximately 150 Å diameter comprising the STAS (Sulphate Transporter and Anti-anti Sigma factor) domain protein RsbS and (the two-domain) RsbR. The latter consists of an N-terminal globin-like domain and a C-terminal STAS domain,[6,10-12] which is similar to RsbS. The structure we determined by cryo-EM single particle reconstruction shows that the STAS domains of these proteins interact to form a pseudo-icosahedral core consisting of 10 dimers of RsbS and 20 dimers of RsbR with the N-terminal domains of RsbR projecting from this core in an arrangement with D2 symmetry (Fig. 1). The presence of a number of RsbR paralogues in *B. subtilis* and the fact that at least one of these, YkoB, can displace RsbR in vitro implies that the structure is dynamic in vivo.[5] Stressosomes may contain a single RsbR paralogue, or a combination of all of them in various cell cycle dependant combinations; our current data indicates they can consist minimally of RsbR with RsbS acting as a scaffold, and that RsbR and YkoB can interact in in vitro reconstituted stressosomes.[5]

The protein kinase RsbT is sequestered by RsbS in the stressosome as part of a ternary pre-signaling complex.[13] Upon the receipt of stress, as perceived by the N-terminal domain of an RsbR paralogue, RsbT phosphorylates RsbS and RsbR and dissociates to activate the $\sigma^B$ cascade.[6,14] In our work, we visualized the ternary RsbR:RsbS:RsbT complex and show that RsbT sits above RsbS, poised for an activating signal to allow it to catalyze the phosphorylation of the STAS domains of RsbS and RsbR.[15] In the case of

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RsbR, the activating signal is currently unknown; the N-terminal domain of this protein adopts an interesting non-heme globin fold,[10] but no ligand has yet been identified. The RsbR paralogue, YtvA, has an N-terminal LOV domain that responds to blue light.[8,9,16] When illuminated with blue light, crystals of the LOV domain of YtvA show distinct conformational changes in the C-terminal alpha-helix that links the two domains of the protein.[16] It can be extrapolated from the obvious constraints of the crystal lattice that this alteration in structure may be greater in magnitude when the native protein is in solution. We thus propose that upon ligand binding, or activation, structural changes in the N-terminal domains of the RsbR paralogues are transmitted to STAS domains that lead to a rearrangement of the core of the complex to permit RsbT to phosphorylate the proteins and dissociate.

That RsbS and RsbR and its paralogues should act together to sequester RsbT in a supra-molecular complex leads to two questions that we address in our work: do stressosomes represent stable complexes in vivo and does the formation of the complex imply some co-operativity in the signaling outcome? To address the first question we used immunofluorescence to localize RsbR within *B. subtilis* cells. The results showed that the fluorescence was present in about 20 discrete extra-nuclear foci, consistent with the formation of large complexes in the cytoplasm. These foci were shown to be resistant to ethanol stress, which indicates the RsbR complexes are stable throughout the duration of the stress-response. The second question was addressed by performing a Miller assay on a $\sigma^B$ dependent *ctc-lacZ* reporter gene fusion in a strain of *B. subtilis* to quantify $\sigma^B$ activity as a function of the concentration of the $\sigma^B$ agonists, ethanol and NaCl. Our results showed that the response to these agonists were sigmoidal, indicating that the response to environmental stress is co-operative in at least one point of the cascade. The Hill co-efficient for both experiments was ~8; by contrast, the Hill co-efficient of hemoglobin, the prototypical example of cooperative behavior, is around 3. The stressosome, with the multiple sensors and multiple copies of the activating kinase RsbT, seems to represent the most likely point at which the system would show co-operativity. Furthermore, the energy stress response, which activates the $\sigma^B$ cascade at a point downstream of the stressosome, does not show any co-operativity, supporting our view that the stressosome is responsible for the co-operativity in the system.

In summary, we determined the pseudo-atomic structure of the stressosome by fitting X-ray crystallographic models into a medium-resolution cryo-EM derived molecular envelope. This structure presents a novel adaptation to integrating diverse signals to a single outcome with the potential for some degree of co-operativity as shown by our LacZ assays. The localization of the stressosome was visualized in *B. subtilis* and is consistent with the level of expression of the constituent proteins and their presence within supra-molecular complexes.

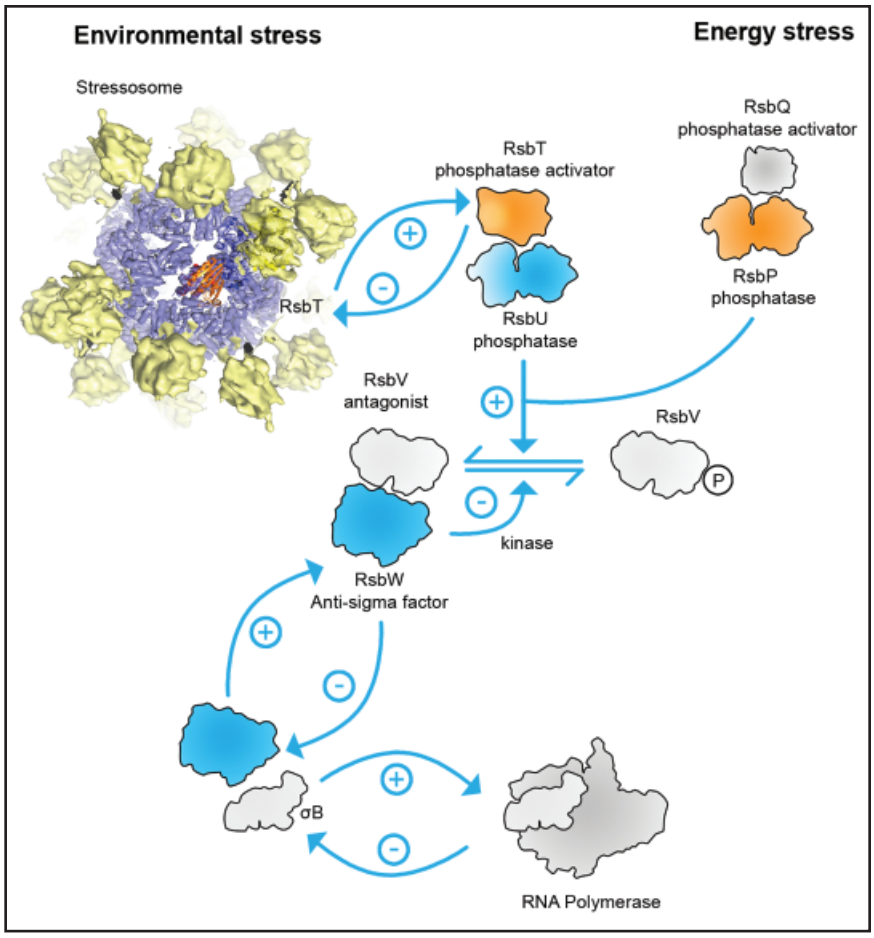


Figure 1. The $\sigma^B$ cascade of *B. subtilis*. A schematic representation of the $\sigma^B$ cascade with the electron microscopy derived structure of the stressosome shown as a surface representation, with the N-terminal domains of RsbR shown in yellow and the core (consisting RsbS and the C-terminal domains of RsbR) in blue. A single molecule of RsbT is shown as an orange secondary structure cartoon.

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